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Gastrothylax crumenifer: Ultrastructure and histopathology study of in vitro trematodicidal action of *Marattia fraxinea* (Sm.)

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Abstract

Background: Trematodicidal action of *Marattia fraxinea* (Sm.) rhizome, extracts against adult *Gastrothylax crumenifer* and its morphological changes under light and scanning electron microscope was studied in this work. This plant according to folk medicine has been reported to be used as antioxidant, antimicrobial and anthelmintic against ankylostomiasis.

Methods: Preliminary screening involved the qualitative methods to detect the presence tannins, saponins, quinones, terpenoids, steroids, flavonoids, phenol, alkaloids, glycosides, cardiac glycosides, coumarins, anthocyanin and betacyanin. Total phenol, total terpenoids, total tannin and total flavonoids were quantitatively estimated. Total phenolic content was estimated by Folin- Ciocalteau method. Total flavonoid content was determined by the aluminium chloride colorimetric method. In-vitro incubation study of *Marattia fraxinea* Sm. extracts against *Gastrothylax crumenifer* were performed using 25 ml of Hedon-Fleig (H-F) salt solution containing various concentrations (1, 2, 3, 4 and 5 mg/ml) respectively as test extracts, positive control Oxyclozanide @ 1% (250 mg/25 ml) and negative control H-F salt solution, distributed to 7-Petri plates in an incubator with 5% CO_2 at 37 °C. Twenty five amphistomes were incubated and the motility of control and test group was observed under dissection microscope at a regular time interval of (0, 10, 15, 30 and 60) min respectively. The motility response of the parasites was categorized with specific score 3, 2, 1, 0 respectively. Relative Motility (RM) value and Lethal Concentration 50 (LC_{50}) were determined by probit regression analysis. Ultrastructure and histopathological changes on morphology of *G.crumenifer* were interpreted. The bioactive compounds were analysed using gas chromatography-mass spectrometry (GC-MS) instrument.

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Results: Phytochemical analysis of *M.fraxinea* (Sm.) revealed the presence of secondary metabolites tannins, saponins, quinones, terpenoids, steroids, flavonoids, phenol, alkaloids, cardiac glycosides, coumarins, and betacyanin. Total terpenoid, total phenol, total tannin and total flavonoid content were found to be 16.00 ± 0.85 mg/g. 5.40 ± 0.71) mg GAE/g, 7.90 ± 0.24 mg TAE/g and 1.25 ± 0.41 mg QE/g respectively. GC-MS analysis showed presence of PHYTOL, a diterpenoids, fern-8-ene, a triterpenoids shown to have potent anthelmintic property. In vitro incubation study revealed death of all trematodes, lethal at 60 min incubation time at 5 mg/ml concentration, indicated Relative Motility (RM) value is 0, least effective compared to positive control Oxyclozanide, where RM value is 0 at 10 min. Lethal concentration 50 (LC_{50}) was found to be 4.873 mg/ml. Confirmative study on gross morphology, histopathology, and ultra-structural changes showed considerable effect on suckers, teguments and internal organs and were dose dependent.

Conclusion: It is indicative that presence of terpenoids, flavonoids, tannins and phenolics which are contributing factors for anthelmintic activity, producing various degree of damage to suckers, teguments and internal organs. Hence *M. fraxinea* (Sm.) can be a lead for synthesis of a new trematodicidal drug as alternative for other drugs and overcome anthelmintic resistance and cost effective.

Keywords: *Marattia fraxinea* Sm, Phytochemistry, GC-MS analysis, *Gastrothylax crumenifer*, Anthelmintic

Background

Helminthiasis is one of the major problem in livestock caused by parasitic diseases viz., trematodes, cestodes and nematodes. Trematodiasis is often an important problem in small ruminants most probably in the tropical and sub-tropical countries causing heavy mortality and morbidity among livestock. The anthelmintic used against helminth infection is of major concern, since most of them develop resistance against parasites *Haemonchus contortus, Fasciola hepatica* and various trematode infection. The anthelminth (Oxyclozanide) a salicylanide compound commonly used as flukicide in veterinary practice developed resistance against amphistomes of sheep due to repeated and improper use of anthelmintic for the worm control programme [1]. The present study was conducted to compare the efficacy of oxyclozanide and *Marattia fraxinea* Sm. against trematode model, *Gastrothylax crumenifer*.

Paramphistomosis is a parasitic infection of the domestic and wild ruminants caused by trematodes belonging to the family Paramphistomidae causing economically considerable problems affecting livestock industry by reducing the production [2]. When immature, the flukes live in the small intestine and abomasum, from where they move to the rumen and become adults. As other digeneans, paramphistomes require snail to complete the life cycle. Snails of the families Planorbidae, Bulinidae and Lymnaeidae acting as intermediate hosts [3].

The pathogenesis primarily caused by immature stages, which are embedded in the mucosa and attaching mucosa by drawing piece of mucosa in to suckers causing necrosis and haemorrhages [4]. It causes an acute gastroenteritis and anaemia with high morbidity especially in young animals, particularly small ruminants [4, 5]. *Gastrothylax crumenifer* is one of the amphistome, has a world-wide distribution with the highest prevalence in subtropical and tropical areas of Africa, Europe, Russia, Australia and also in South and Southeast Asia [6]. Chemotherapy is the widely accepted control method however, emergence of resistant strains and increasing concern about drug residues in the food chain have highlighted the need for alternative control strategies. Traditional plant based eco-friendly medicines offer an alternative to chemical based anthelmintics and are reported high percentage of cure with a single therapeutic dose [7]. The trials to using plants as anthelmintic remedies go back to older methods of the pre chemotherapeutical periods; however they have become more and more important today [8]. Several plants have been tested for their anthelmintic efficacies [9–18], against trematode infection. To date, there has been no literature cited to show the anthelmintic property of *Marattia fraxinea* Sm., lower vascular cryptogamic plants scientifically investigated to establish whether or not they have antitrematodal properties in vitro.

Marattia fraxinea Sm. (family: Marattiaceae) commonly known as tree fern and known for its medicinal properties which is used as a remedy for ankylostomiasis (Nematode Parasite) in Usambara and South Africa [19]. They are shade loving plants seen under high altitude regions of the world. They have tremendous value in folklore medicine and are widely used as an anthelmintic by the tribal community of Western Ghats region of India.

Most of the parasites have become resistant to chemotherapy, so alternatives are urgently required. Since vaccination has failed in most instances, the search for small molecules is still an option. For malaria and trypanosomiasis quite a number of medicinal plants and isolated natural products have already been tested, but

for most of the other parasitic diseases such information is largely missing. Most of the anti-parasitic properties of extracts and isolated natural products have been tested in vitro only. Translation of the in vitro research results into in vivo trials is urgently required. Furthermore, even if animal experiments were successful, we would need clinical trials of the new compounds alone or in combination with established parasiticidal drugs to prove their efficacy and safety. These developments are costly and it is presently difficult to attract the pharmaceutical industries into these fields for various reasons [20]. The objective of the present work was to investigate the in vitro anthelmintic efficacy of solvent extracts of *M.fraxinea* Sm. and subsequent study on morphological changes towards trematode model *Gastrothylax crumenifer*.

Methods

Collection and preparation of extract

Marattia fraxinea Sm. (Fig. 1a) belongs to family "Marattiaceae" commonly known as "tree fern, pitted potato fern" (Synonyms: *Myrrotheca fraxinea* and *Prisana fraxinea* (Sm.) Murdock) distributed in Asia, Africa, Australia and most of tropical and sub-tropical regions of the world. The plant is terrestrial mostly confined to moist conditions along perennial streams and at waterfalls, in deep shade in evergreen forests. Nanophanerophyte, mesophyte; fronds mesomorphic. Not edaphically bound. The ferns were collected from Upper Kodhayar region of Western Ghats, Kanyakumari district, India identified [21] and authenticated [22] at Scott Christian College, Nagercoil, India and a voucher specimen (Fig. 1b) (SPCH 1006) was deposited at A.Veeraiya Vandayar Memorial Sri Pushpam College, Thanjavur district, India. The extract was prepared as per the earlier method [23]. Briefly, one gram of dried powder rhizomes of *M. Fraxinea* Sm. plant materials were extracted with 75% ethanol, acetone, chloroform, aqueous and petroleum ether (Merck, extra pure) for 1 min using an Ultra Turax mixer (13,000 rpm) and soaked overnight at room temperature. Then the mixer was filtered through Whatman No. 1 paper and evaporated at 40 °C to a constant weight. The extract was dissolved in respective solvents and stored at 18 °C until use.

Phytochemical analysis

Preliminary screening involved the qualitative methods [24] to detect the presence tannins, saponins, quinones, terpenoids, steroids, flavonoids, phenol, alkaloids, glycosides, cardiac glycosides, coumarins, anthocyanin and betacyanin. Total phenol, total terpenoids, total tannin and total flavonoids were quantitatively estimated. Total terpenoid content was estimated using the method of [25], total tannin content was determined using the procedure [26], total phenolic content was estimated by Folin- Ciocalteau method [27] and total flavonoid content was determined by the aluminium chloride colorimetric method [28].

Column chromatography

Column chromatography was performed using slurry method [29]. Column was packed with slurry of silica gel (mesh size, 60–120) with chloroform. Then dried methanol extract (4 mg) of *M. fraxinea* Sm. was first dissolved in methanol and carefully applied by pipette at the top of prepared column. Immediately after application of sample, a gradient of chloroform and methanol (mobile phase) was used as eluent to collect fractions of methanol extract of *M. fraxinea* Sm. The flow rate of the column is determined by the packing method. To speed up the process, elute all of the solvent using compressed air and allow air to flow through the column for approximately 2 h. The column was run with a gradient of chloroform : methanol (98:2, 95:5, 90:10, 80:20, 70:30, 50:50, 30:70, 20:80, 10:90, 5:95, 2:98) finally 100% methanol and 13 fractions (F1-F13) were collected (1–3 mL).

Fig. 1 *Marattia fraxinea* (Sm.) **a**: Real image. **b**: Herbarium

Thereafter, from all the collected fractions solvent was removed by evaporation at room temperature. After evaporation of solvent from the fractions F4, colourless crystals were isolated. The crystals of the fractions were first separately treated with Petroleum ether and then filtered. The crystalline residues were then retreated with chloroform and were recovered after filtration. The filtered fraction was further used for Gas chromatography-mass spectrometry analysis and for in vitro incubation study against *G.crumenifer*.

Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis was performed as per [30] in separated fraction of fern extract under column chromatography. Chemical analysis was performed using a gas chromatograph (Trace Ultra GC) interfaced with a quadrupole mass spectrometer (DSQ II) (Thermo Scientific Co.), Chromatographic separation was achieved with DB-35 MS fused silica capillary column (30 m X 0.25 mm ID X 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA). Helium with a purity of 99.999% was used as the carrier gas at a flow rate of 1.0 mL min – 1. One μL of extract was injected in splitless mode using an auto sampler. The injector port, interface and ion source temperatures were set at 250, 260 and 250 °C, respectively. The GC temperature was programmed as follows: 75 °C (2 min), 10 °C min – 1 ramp to 150 °C, 5 °C min – 1 ramp to 250 °C, and 10 °C min – 1 ramp to 260 °C (10 min hold). The mass spectrometer was operated in electron ionization (EI) mode at 70 eV and at an emission current of 60 μA. Full scan data was obtained in a mass range of m/z 50–650. Scanning interval and SIM sampling rate were 0.5 and 0.2 s, respectively. The mass selective detector was operated in Total Ion Current monitoring (TIC) mode. The identification of peaks was based on the use of reference standards and NIST11 and Wiley 9 library. The mass spectra obtained were inspected manually and only those molecules with probability matching higher were considered.

Collection and identification of trematodes

Adult live amphistomes were collected from the rumen of infected sheep and goats slaughtered at Orathanadu, Thanjavur and Pattukottai abattoir, Tamilnadu, India. Adult live worms were washed thoroughly in physiological saline and maintained in Hedon-Fleig solution (pH 7.0) [31]. The worms were identified as *Gastrothylax crumenifer,* based on morphology [4] and subjected for in vitro incubation study.

In vitro incubation study

The purified extract/elute of *M.fraxinea* Sm. with greater antioxidant potential OD values was used for the in vitro incubation study of *G. Crumenifer* in different concentration. Hedon-Fleig (H-F) salt solution (NaCl 119.82 mM, KCl 4.01 mM, $MgSO_4$ 0.29 mM, $CaCl_2$ 0.40 mM, $NaHCO_3$ 17.8 mM, glucose 22.3 mM) was prepared by the method of [32]. The various concentrations (1 to 5 mg/ml) of test extract was prepared in 25 ml of H-F salt solution. The adult parasite was incubated in extract containing H-F solution with antibiotic streptomycin sulphate (6900 unit 10 mg/I) and benzyl penicillin (9900 units/I) at 38 ± 1 °C in BOD incubator. Oxyclozanide (OXY) 1% (250 mg/25 ml) I.P. (vet) 3.4% w/v Pack (Vetco Pharma, India) used as positive control and only H-F salt solution as negative control. Adult parasites were randomly selected and 25 flukes each for all concentrations, positive and negative controls in triplicate were incubated at 37 °C in CO_2 incubator with 5% CO_2. The motility of the control and drug treated flukes were assessed by examination under a dissection microscope at 0, 10, 15, 30 min and 1 h of incubation. The motilities of the flukes at each incubation period were scored using the criteria proposed by [33]. The dead flukes were assessed by disturbing the flukes using a needle. Motility score was assigned by using the following criteria,

3 = movement of the whole body
2 = movement of only part of the body
1 = immobile but not dead
0 = immobile and dead

Parasitological and histopathological study

The morphological and histopathological variation of the dead flukes were studied under stereo-zoom, light and scanning electron microscope for correlation and comparison.

Flukes from the groups with lowest (1 mg/ml), highest concentrations (5 mg/ml) of the fern extract, positive control and negative control were subjected for carmine staining for gross morphological study. The amphistomes were washed thoroughly with 0.1 M phosphate buffer saline, pH 7.4 and pressed in between two slides, tied both slides with rubber band and submerged in formalin for at least 12 h for fixing, then they were washed in running tap water overnight. The washed amphistomes were dehydrated with 70% alcohol three times and stained with acetic carmine dye overnight. The amphistomes were destained with 1% acid alcohol, washed in ammonia water, dehydrated with graded series of ethanol, cleared in xylene and permanently mounted with DPX [4, 34]. The stained specimens were examined for abnormalities under stereo-zoom and light microscope and photographed.

After incubation worms from the groups as above were were stained using haematoxylin and eosin for histopathological changes [35]. The worms were fixed in

10% formaldehyde for 24 h, dehydrated with ascending series of ethanol and cleared with xylene. They were then embedded in paraffin, sectioned longitudinally and transversely at thickness of 5 μm, stained with haematoxylin and eosin [36] and examined for abnormalities under light microscope and photographed.

Scanning Electron Microscope (SEM) study

After incubation worms from the groups as above were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer solution (PBS), pH 7.4 for 12 h at 4 °C. Samples were washed in phosphate buffer 3 times, and post fixed in 1% osmium tetroxide (OsO_4) in 0.1 M PBS, pH 7.4 at 4 °C for 2 h [37]. They were washed repeatedly in cold double distilled water three times, dehydrated through an ascending concentrations of ethanol, dried in a Hitachi HCP-2 critical point drying machine using liquid CO_2, mounted on aluminium stubs, coated with gold-palladium in a JEOL JSM-5610LV INCA EDS ion-sputtering apparatus set at 10 mA for 9 min. Specimens were observed and photographed at various magnifications using JEOL JSM-5610LV INCA EDS scanning electron microscope operating at 0.5 to 30 kV.

Statistical analysis

The efficacies of the tested drugs against adult *G. crumenifer* were based on calculated Relative Motility (RM) values using the formula listed below [33].

$$\text{RM Value} = \frac{\text{MI test} \times 100}{\text{MI control}}$$

$$\text{Motility Index (MI)} = \frac{\Sigma nN}{N}$$

n = motility score, N = number of flukes with the score of n.

Relative Motility (RM) values is indicated to assess the motility and viability of flukes to determine the mortality pattern of trematode model under study [11, 37, 38]. A smaller RM values indicates higher mortality rate. Then finally the (LC_{50}) that was able to kill half of the population was determined by probit regression analysis [39].

Results

Phytochemical analysis of various extracts

Qualitative phytochemical analyses indicated the presence of secondary metabolites tannins, saponins, quinones, terpenoids, steroids, flavonoids, phenol, alkaloids, cardiac glycosides, coumarins, and betacyanin. However Glycosides and anthocyanin were absent in the extracts studied. Out of 5 solvent extracts, acetone extract performed well to show positivity of phytoconstituents than other 4 solvent extracts (Table 1). The quantitative estimation of the phytochemicals have shown that *M. fraxinea* Sm. contained higher amount of terpenoid (16.00 ± 0.85 mg/g) followed total phenol (5.40 ± 0.71) mg GAE/g), total tannin (7.90 ± 0.24 mg TAE/g), and total flavonoid (1.25 ± 0.41 mg QE/g) (Table 2).

Table 1 Qualitative phytochemistry of various solvent extract of *Marattia fraxinea* Sm.

Phytoconstituents	Aqueous	Acetone	Chloroform	Ethanol	Petroleum ether
Tannins	-	++	-	++	-
Saponins	+	++	+	-	-
Quinones	++	++	+	+	-
Terpenoids	-	++	+	++	+
Steroids	-	++	+	+	+
Flavonoids	++	++	-	++	-
Phenol	++	++	+	++	+
Alkaloids	-	++	-	+	-
Glycosides	-	-	-	-	-
Cardiac glycosides	_	+	+	+	+
Coumarins	+	++	-	+	-
Anthocyanin	-	-	-	-	-
Betacyanin	+	+	-	+	-

+ = Positive, ++ = Strong positive, - = Negative

GC-MS analysis

The GC-MS chromatogram and Mass spectrum of eight chemical compounds were mentioned in (Tables 3 and 4, Fig. 2). The GC-MS analysis showed predominant peak in presence of terpenoids and polyphenols derivatives.

Identification of the parasite

In the carmine staining worms showed an anteriorly opening ventral pouch extending the whole ventral surface, a posterior terminal sucker, intestinal caeca that extends up to the level of lobed horizontal testes and also showed the crossing of uterus from right to left. Thus it was confirmed as *Gastrothylax crumenifer* (Fig. 3).

In vitro incubation study

The results of in vitro incubation study depicted in (Table 5 and Fig. 4). At 1 mg/ml, the relative motility at 30 min was 0.64 which indicated 36% of the flukes were dead while at 2 mg/ml, the relative motility at 30 min

Table 2 Quantitative phytochemical analyses of *Marattia fraxinea* Sm.

Terpenoid mg/g	Total Phenol content (mg GAE/g)	Total Flavonoid content (mg QE/g)	Total Tannin content (mg TAE/g)
16.00 ± 0.85	5.40 ± 0.71	1.25 ± 0.41	2.92 ± 0.24

Table 3 Secondary metabolite detected in whole plant methanol extracts of *Marattia fraxinea* Sm.

No	RT	Name of the compound	Molecular formula	MW	Peak area %
1	5.26	1-Hexanol, 2-ethyl-(CAS)	$C_8H_{18}O$	130	0.71
		2 ETHYL HEXANOL	$C_8H_{18}O$	130	0.71
2	7.93	4-(1-hydroxy-ethyl) ç butanolactone	$C_6H_{10}O_3$	130	4.06
		DIHYDROTHIOPHENE	C_4H_6S	86	4.06
		Thiophene, 2,3-dihydro- (CAS)	C_4H_6S	86	4.06
3	16.02	1,2,3-Propanetricarboxylic acid, 2-hydroxy-, triethyl ester (CAS)	$C_{12}H_{20}O_7$	276	17.80
		Triethyl citrate	$C_{12}H_{20}O_7$	276	17.80
4	18.08	Phenol, 2-methyl-5-(1,2,2-trimethylcyclopentyl)-, (S)- (CAS)	$C_{15}H_{22}O$	218	0.69
		Xanthorrhizol	$C_{15}H_{22}O$	218	0.69
5	22.26	Dibutyl phthalate	$C_{16}H_{22}O_4$	278	53.07
		1,2-Benzenedicarboxylic acid, dibutyl ester (CAS)	$C_{16}H22O4$	278	53.07
6	25.15	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]- (CAS)	$C_{20}H_{40}O$	296	0.44
		Phytol	$C_{20}H_{40}O$	296	0.44
7	29.70	Hexanedioic acid, bis(2-ethylhexyl) ester (CAS)	$C_{22}H_{42}O_4$	370	0.95
		Hexanedioic acid, dioctyl ester (CAS)	$C_{22}H_{42}O_4$	370	0.95
8	33.26	1,2-Diphenyl-5-(t-butyl)acephenanthrylene	$C_{32}H_{26}$	410	2.83
		6á-Methoxy-3à,5à-cyclofurosta-16,20,(22)-diene	$C_{28}H_{42}O_2$	410	2.83
		Fern-9(11)-ene	$C_{30}H_{50}$	410	2.83

Table 4 Activity of Phyto-components identified in whole plant methanol extract of *Marattia fraxinea* Sm.

Name of the compound	Molecular formula	Compound nature	Activity
1-Hexanol, 2-ethyl-(CAS) 2 ETHYL HEXANOL	$C_8H_{18}O$ $C_8H_{18}O$	Fatty alcohol	Antifungal, herbicide
DIHYDROTHIOPHENE Thiophene, 2,3-dihydro- (CAS)	C_4H_6S C_4H_6S	Heterocyclic aromatic compound	Anthelmintic, antioxidant
1,2,3-Propanetricarboxylic acid, 2-hydroxy-, triethyl ester (CAS) Triethyl citrate	$C_{12}H_{20}O_7$ $C_{12}H_{20}O_7$	Fatty acid derivative	Antiulcer, Inhibit oxidative phosphorylation, kreb's cycle and anti-inflammatory
Phenol, 2-methyl-5-(1,2,2-trimethylcyclopentyl)-, (S)- (CAS) Xanthorrhizol	$C_{15}H_{22}O$ $C_{15}H_{22}O$	Polyphenol derivatives Terpenoid	Antioxidant, anti-inflammatory and antibacterial anthelmintic activity Anticancer, antimicrobial, anti-inflammatory, anthelmintic antioxidant,antihyperglycemic, antihypertensive, antiplatelet, nephroprotective, hepatoprotective, estrogenic and anti-estrogenic effects.
Dibutyl phthalate 1,2-Benzenedicarboxylic acid, dibutyl ester (CAS)	$C_{16}H_{22}O_4$ $C_{16}H22O4$	Plasticizer compound	Anti-fouling, antimicrobial
2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]- (CAS) Phytol	$C_{20}H_{40}O$ $C_{20}H_{40}O$	Diterpene Acyclic Diterpene Alcohol	Antioxidant, anticancer, antimicrobial, anthelmintic Antimicrobial, anthelmintic anticancer, anti-inflammatory, diuretic and antidiabetic
Hexanedioic acid, bis(2-ethylhexyl) ester (CAS) Hexanedioic acid, dioctyl ester (CAS)	$C_{22}H_{42}O_4$ $C_{22}H_{42}O_4$	Saturated fatty acid	antioxidant, hypocholesterolemic, nematicidal, pesticidal, lubricant, haemolytic, cosmetic, antiandrogenic and flavouring property.
1,2-Diphenyl-5-(t-butyl)acephenanthrylene Fern-9(11)-ene	$C_{32}H_{26}$ $C_{30}H_{50}$	Aromatic compounds/ flavonoids Fernane type triterpenoids	Cancer-preventive, flavour Analgesic, antinociceptive, anti-implantation, anti-inflammatory, antiviral, anthelmintic property.

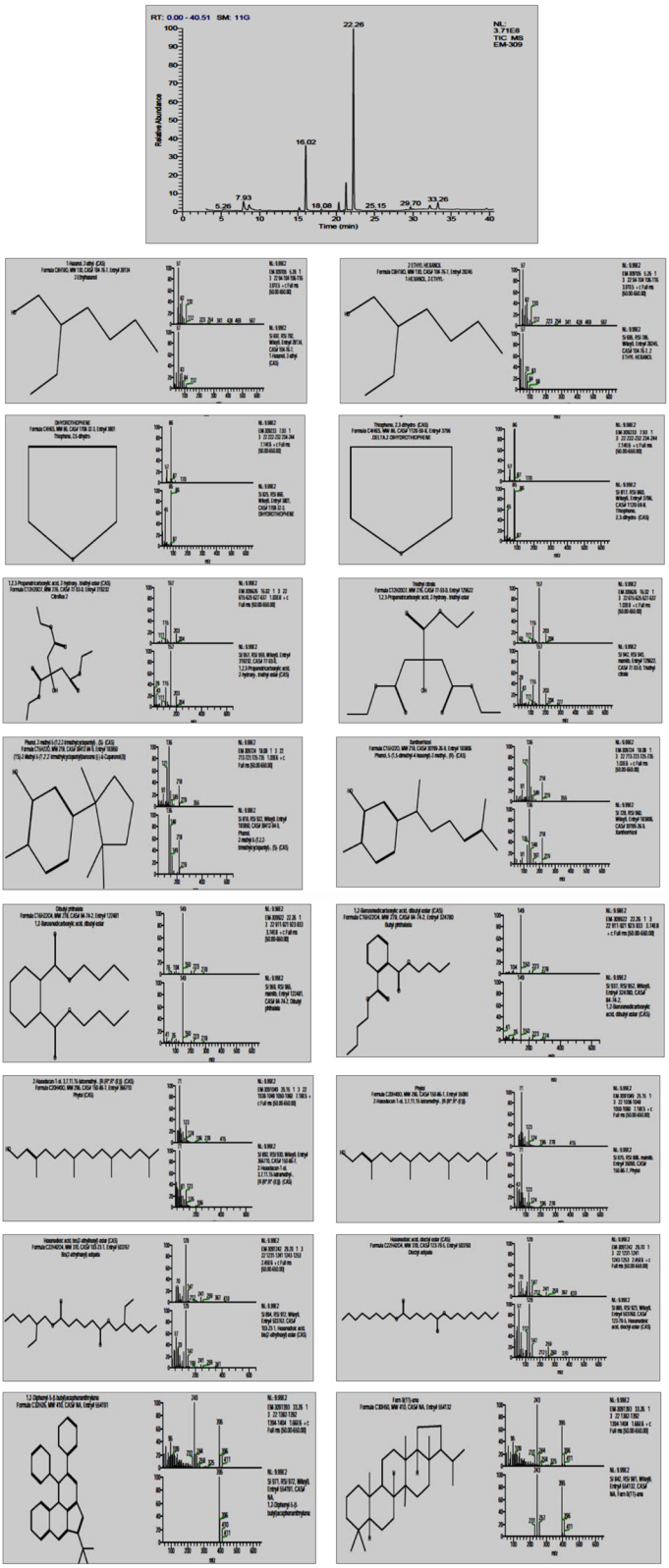

Fig. 2 GC-MS chromatogram of methanol extract of whole fern of *Marattia fraxinea* Sm.

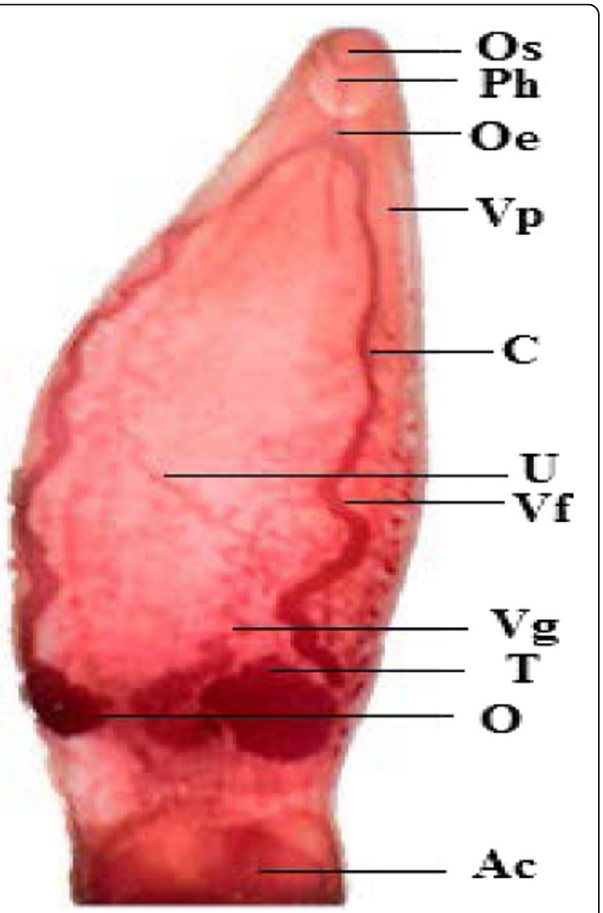

Fig. 3 General morphology of *Gastrothylax crumenifer* (Negative control). Oral sucker (Os), Pharynx (Ph), Oesophagus (Oe), Ventral pouch (Vp), Caecum (C), Uterus (U), Vitelline follicle (Vf), Vitelline gland (Vg), Testes (T), Ovary (O) and Acetabulum (Ac)

was 0.51 which means 49% of the flukes were dead while at (3, 4 and 5) mg/ml, the relative motility at 30 min was 0.37, 0.17 and 0.04 respectively indicated 63, 83 and 96% flukes were dead. However positive control (250 mg/25 ml) at 10 min merely killed all the worms studied under incubation trials (Table 5) indicative Positive control is better efficient than test groups. The higher concentration (5 mg/ml) test extracts kill all the worms at 60 min of incubation time. Using probit regression analysis in determining the LC_{50}, 10 min time was the most suitable one. The results of the entire study could be presented as such, based on which 10 min timing could be expressed as the best one for estimation of LC_{50}. (Figure 4) stated probit regression was significant. Chi-square test for heterogeneity indicated adequacy of probit model to the data. The concentration for 50% death (LC_{50}) was 4.873 with a 95% confidence interval (CI) of 4.235 to 6.038 (Tables 6 and 7). Using probit regression analysis in determining the LC_{50} (Fig. 4), results from the viability and motility assay showed that 4.873 mg/ml was the lethal concentration that was able to kill half of the population of flukes in the experiment.

Gross morphological study

The morphological changes of fern extracts treated and control group were studied under stereo-zoom microscope. Control group showed no morphological changes (Fig. 3). Lower concentration drug showed least changes when compared to high concentrated drug. Standard control showed degenerative changes towards Oral sucker, Acetabulum, teguments and testes (Fig. 5). Test group of *M. fraxinea* Sm. extract showed significant degenerative changes in Oral sucker, Acetabulum, tegument, shrinkage of testes and degeneration of uterus (Fig. 5). No changes were notified in other internal organs like intestines of flukes both in test and standard control group.

Histopathology

The histological sections of negative control showed normal features of teguments, oral sucker, acetabulum, testes and Vitelline gland. However standard control and test group showed significant changes in surface syncytium, tegument cells and rupture of parenchymatous cell, mild vacoular degeneration of teguments, degeneration of testes and Vitelline gland (Fig. 6).

Scanning electron microscope study

In the control group, *G. crumenifer* has a pouched body with anterior end bounded by thick muscular rim. The tegument is composed of transverse major folds alternating with major grooves. No tegumental changes were observed in control flukes (Fig. 7). In oxyclozanide treated flukes, there were retracted changes towards Oral sucker, Acetabulum, Papillae around Oral sucker showing blebs, erosion of tegument result of ruptured blebs (Fig. 7). *M.fraxinea* Sm. extract treated flukes showed anterior sucker showing degenerative changes at 1 mg/ml conc, Severe Blebbing on tegumental ventral (5 mg/ml) conc, deep furrows and major tegumental folds indicating disrupted papillae on ventral surface (5 mg/ml) conc, middle portion of ventral surface shows prominent deep furrows between major folds criss-crossed by minor folds and grooves (5 mg/ml) (Fig. 7).

Discussion

Acetone based extraction showed better phytochemicals from fern. Tannin, Phenol, Terpenoid, Flavonoid, Quinones, steroids, alkaloids and saponins showed strong positivity in acetone fern extract which was the best solvent to express phyto-constituents in this study. Mithraja et al. [40], performed phytochemical screening with acetone, benzene, chloroform, ethanol, petroleum ether and aqueous extracts of whole plants of

Table 5 Motility index and relative motility values of *Gastrothylax crumenifer* on various time mode of exposure to *Marattia fraxinea* Sm. extract

Conc (mg/L)	0 min			10 min			15 min			30 min			1 h		
	Replicates		Average RM	Replicates		Average RM	Replicates		Average RM	Replicates		Average RM	Replicates		Average RM
	MI	RM		MI	RM		MI	RM		MI	RM		MI	RM	
0 (NC)	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00
	3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00	
	3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00	
1	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	2.96	0.99	0.97 ± 0.009	1.96	0.65	0.64 ± 0.007	1.16	0.39	0.41 ± 0.012
	3.00	1.00		3.00	1.00		2.88	0.96		1.88	0.63		1.28	0.43	
	3.00	1.00		3.00	1.00		2.92	0.97		1.96	0.65		1.20	0.40	
2	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	2.84	0.95	0.92 ± 0.024	1.56	0.52	0.51 ± 0.013	1.04	0.35	0.33 ± 0.009
	3.00	1.00		3.00	1.00		2.80	0.93		1.56	0.52		0.96	0.32	
	3.00	1.00		3.00	1.00		2.60	0.87		1.44	0.48		1.00	0.33	
3	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	2.76	0.92	0.88 ± 0.021	1.16	0.39	0.37 ± 0.007	0.60	0.20	0.19 ± 0.015
	3.00	1.00		3.00	1.00		2.60	0.87		1.08	0.36		0.48	0.16	
	3.00	1.00		3.00	1.00		2.56	0.85		1.12	0.37		0.64	0.21	
4	3.00	1.00	1.00 ± 0.00	1.84	0.61	0.59 ± 0.011	1.32	0.44	0.43 ± 0.003	0.56	0.19	0.17 ± 0.007	0.20	0.07	0.06 ± 0.007
	3.00	1.00		1.72	0.57		1.28	0.43		0.52	0.17		0.20	0.07	
	3.00	1.00		1.76	0.59		1.28	0.43		0.48	0.16		0.16	0.05	
5	3.00	1.00	1.00 ± 0.00	1.44	0.48	0.46 ± 0.012	0.84	0.28	0.25 ± 0.017	0.12	0.04	0.04 ± 0.006	0.00	0.00	0.00
	3.00	1.00		1.32	0.44		0.76	0.25		0.08	0.03		0.00	0.00	
	3.00	1.00		1.40	0.47		0.88	0.22		0.16	0.05		0.00	0.00	
OXY (PC)	3.00	1.00	1.00 ± 0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	3.00	1.00		0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
	3.00	1.00		0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	

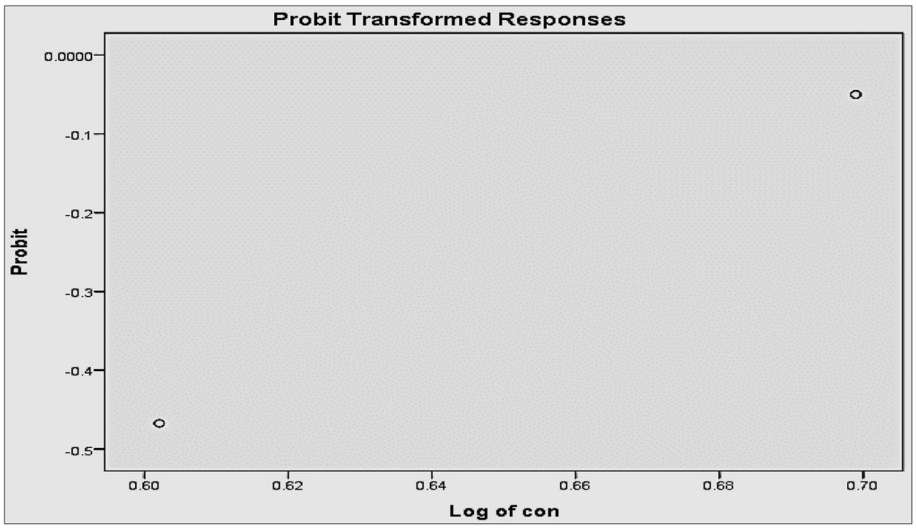

Fig. 4 Lethal Concentration 50 (LC_{50}) using probit regression analysis of *Marattia fraxinea* (Sm.) extract at various doses

B.orientale, C.thalictroides, D.heterophyllum, D.linearis, H.arifolia, L.ensifolia, N.multiflora, P.calomelanos, P.confusa and leaves and rhizomes of *Drynaria quercifolia,* revealed that the presence or absence of the phytoconstituents depend upon the solvent medium used for extraction and the physiological property of individual taxa. The present study on the phytochemical analysis of *Marattia fraxinea* Sm. was in confirmation with the earlier study [40] and stated that tannin containing drugs are used in medicine as astringent and have been found to possess antiviral, antibacterial and anti-parasitic effects for possible therapeutic applications.

Kumudhavalli and Jaykar [41], evaluated the petroleum ether, chloroform, acetone, ethanol and aqueous extracts of the fern *Hemionitis arifolia* (Burm.) Moore, for preliminary phytochemical screening. Our study was subjected for quantifying *Marattia fraxinea* Sm. Gracelin et al. [42], conducted qualitative and quantitative phytochemical analyses in five *Pteris* fern species. Qualitative analysis of methanol extract exhibited positivity for 10 phytochemical tests. Present study on the qualitative analysis of ethanolic, petroleum ether acetone, chloroform and aqueous extract of fern species showed acetone extract performed well to exhibit positivity for secondary metabolites. The quantitative analysis of the extract of *Pteris* species showed flavonoids content were highest followed by alkaloids and phenolic compounds. The amount of tannin and saponin was very low in the fern extract. However present quantitative study showed terpenoids content was highest and flavonoid content was least in *Marattia fraxinea* Sm.

The results of the phytochemical screening and quantitative estimation of the chemical constituents of plant sample have indicated high content of terpenoids, total tannin, total phenol and flavonoids. Terpenes are widespread in nature, mainly in plants as resent constituents of essential oils. Saponin as a group include compounds that are glycosylated steroids, triterpenoids and steroids alkaloids. Many saponin are known to be antimicrobial to inhibit mould, and protect plant from insect attacks. Saponins may be considered as a part of plants defence systems found in plants named phytoanticipins or phytoprotectants [43]. These structurally diverse compounds have also been observed to kill protozoans and helminths, to be antioxidants and also acts as antifungal and antiviral [44, 45]. Xanthorrhizol is a bisabolane type sesquiterpenoid compound posses variety of antimicrobial, antioxidant, anthelmintic activity [46]. Essential oils are formed by aromatic odor as secondary metabolites, composed of terpenes or terpenoids. The cytotoxic activity of essential oils is mostly due to the presence of phenols, aldehydes and alcohols [47] and are effective

Table 6 Parameter estimate

	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	con	8.889	2.400	3.703	.000	4.185	13.593
	Intercept	−6.114	1.575	−3.881	.000	−7.689	−4.538

[a]PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Table 7 Confidence limit

	Probability	95% Confidence Limits for con			95% Confidence Limits for log(con)[a]	
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound
PROBIT	.010	2.667	1.457	3.289	.426	.164
	.020	2.862	1.685	3.462	.457	.227
	.030	2.994	1.846	3.579	.476	.266
	.040	3.096	1.976	3.671	.491	.296
	.050	3.182	2.088	3.750	.503	.320
	.060	3.257	2.187	3.819	.513	.340
	.070	3.325	2.277	3.882	.522	.357
	.080	3.386	2.361	3.940	.530	.373
	.090	3.443	2.439	3.995	.537	.387
	.100	3.496	2.512	4.047	.544	.400
	.150	3.725	2.832	4.283	.571	.452
	.200	3.918	3.101	4.500	.593	.491
	.250	4.092	3.336	4.717	.612	.523
	.300	4.254	3.548	4.942	.629	.550
	.350	4.410	3.740	5.181	.644	.573
	.400	4.563	3.916	5.441	.659	.593
	.450	4.717	4.080	5.725	.674	.611
	.500	**4.873**	**4.235**	**6.038**	**.688**	**.627**
	.550	5.034	4.384	6.385	.702	.642
	.600	5.203	4.529	6.775	.716	.656
	.650	5.384	4.675	7.217	.731	.670
	.700	5.582	4.826	7.728	.747	.684
	.750	5.803	4.986	8.333	.764	.698
	.800	6.060	5.163	9.076	.782	.713
	.850	6.374	5.368	10.042	.804	.730
	.900	6.791	5.630	11.424	.832	.750
	.910	6.896	5.693	11.787	.839	.755
	.920	7.012	5.763	12.197	.846	.761
	.930	7.142	5.840	12.664	.854	.766
	.940	7.289	5.927	13.209	.863	.773
	.950	7.462	6.026	13.860	.873	.780
	.960	7.669	6.145	14.669	.885	.789

[a]Logarithm base = 10

Lethal concentration to kill 50% of population (LC50) is captured in bold

against a large variety of organisms including bacteria, fungi, viruses, protozoa as well as metazoan parasites [48]. Flavonoids are potent secondary metabolites having high anthelmintic activity and the toxicity of most isolated flavonoids in animal cells is very low [49], several ubiquitous flavonoids genistein, kaemferol, rutin, quercetin etc., showed deleterious effects on selected species of parasitic helminths. Flavonoid kaempferol exerted a strong adulticidal activity on *Schistosoma mansoni* [50]. The anthelmintic activity of flavonoids, genistein, isoflavones found in the root extracts of *Flamingia vestita* [51] mediated its action on cellular/molecular targets in mammals. In flatworms they act on tegumental enzymes causing paralysis and death [52]. The approved anthelmintics for trematodes are Oxyclozanide, Praziquatel and Triclabendazole, however the rapid spread of triclabendazole resistance is an important motivation for drug discovery of novel trematodicidal drugs [53]. *Dryopteris filix-mas* (Dryopteridaceae), contains vermicidal phloroglucinols, such as aspidin, deaspidin and filixic acid are active against intestinal cestodes, paralyze the worm's tegument [54]. Pelletierine

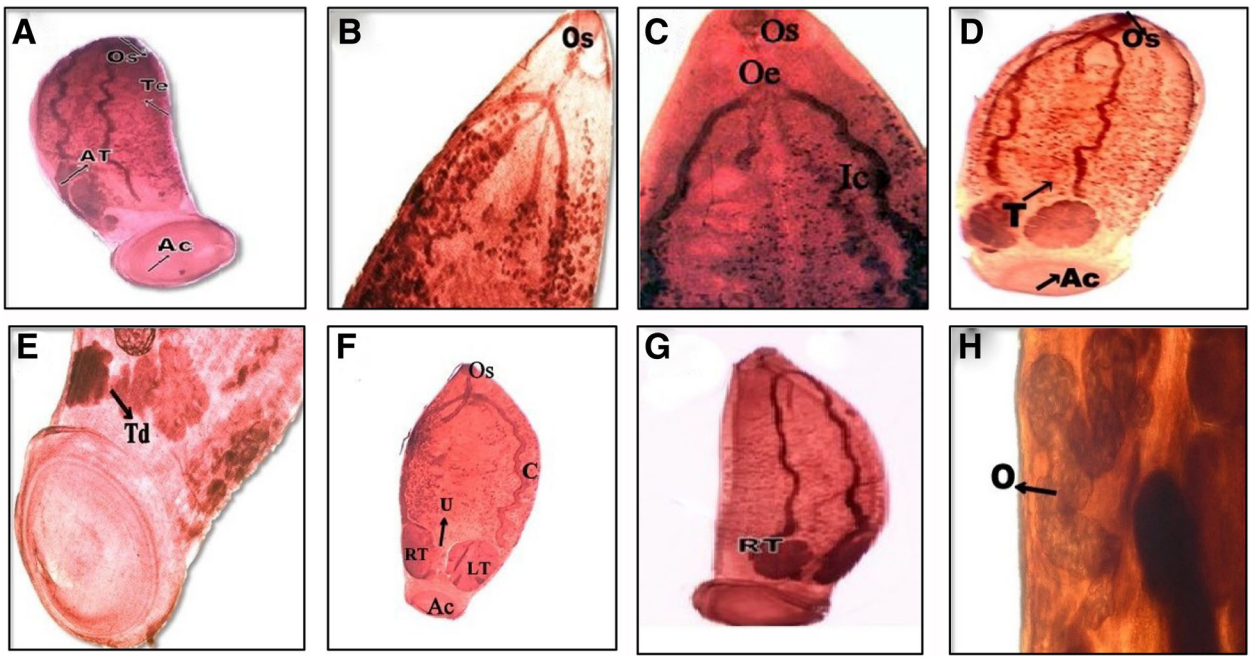

Fig. 5 Gross morphological changes of Standard and treated *Gastrothylax crumenifer*. Stereo Zoom Microscope study of *G.crumenifer* for morphological changes under incubation for 1 h. (**a**-**d** Standard Control) (**a**) Whole view of *G. crumenifer* showing shrinkage of teguments, degenerative changes of Oral sucker (Os). **b** Disruption of Oral sucker (Os). **c** Degenerative changes of Oral sucker (Os). **d** Degenerative changes of Testes (T), Oral sucker (Os) and Acetabulum (Ac). (**e**-**h**: Test Group) (**e**) Degeneration of Acetabulum (Ac) and Testes (Td) at 5 mg/ml conc (**f**) Deformity of Oral sucker (Os), acetabulum and degenerative changes in Right Testes (RT), Left Testes (LT) and Uterus (U) at 5 mg/ml conc (**g**) Severe degenerative changes of Right Testes (RT) at 5 mg/ml conc (**h**) Severe degenerative changes of Vitelline gland, ruptured ovary exposing ova (O) at 5 mg/ml conc.

an anthelmintic alkaloids from *Punica granatum* (Lythraceae) and arecoline from *Areca catechu* (Arecaceae) target acetylcholine receptors [20].

Hrckova and Velebny [55], stated the surface structure of the tegument of trematodes and cestodes represent the potential target sites as the small molecules can be absorbed in the tegument. Athanasiadou and Kyriazakis [56], stated the secondary metabolites, alkaloids in plants act mainly on adult stage of flukes and showed its anti-parasitic effects on some nematode parasites of cattle and goat and also against a digenean fluke of sheep, namely *Paramphistomum cervi.* Several studies showed that extracts from plants grown to serve as human food for example: *coconut, onion, garlic, fig, date, annanas, chicory* have high anthelmintic potential against intestinal nematodes, cestodes and trematodes [8].

2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]- (CAS), PHYTOL, Fern-8-ene, Xanthorrhizol a potent group of terpenoids present in these fern extract under GC-MS analysis might be attributed to significant antioxidant potential and anthelmintic activity. These findings provide quantitative estimation of the phyto-chemicals as well as mineral element analysis which are important in understanding the pharmacological and/or toxicological actions of medicinal plants [57].

Various studies on in vitro and in vivo anti-trematodal activity of medicinal plants viz., alcoholic extract of *Allium sativum* and *Piper longum* [58], *Balanites aegyptica* [17], bark of *Prosopis cineraria* [18], Plumbagin on *Fasciola gigantica* and *Paramphistomum cervi* [37, 59], 50 medicinal plants against *Schistosoma mansoni* and *Echinostoma caproni* used in Côte d' Ivoire [16], *Bombax malabaricum* leaves against *Paramphistomum explanatum* [15], *Dregea volubilis* leaves against *Paramphistomum explanatum* [60], *Artemisia annua, A.absinthium, A.siminatriloba* and *Fumaria officinalis* against adult *Schistosoma mansoni, Fasciola hepatica* and *Echinostoma caproni* in vitro [14], *Aegle marmelos, Andrographis lineata, A.paniculata, Cocculuc hirsutus, Eclipta prostrata* and *Tagetes erecta* against *Paramphistomum cervi* [13], *Flemingia estita* root tuber against *Paramphistomum* sp. [9]. Similarly in vivo study of five allopathic drug on natural fasciolosis infected cattle [61], has been reported. However, Pteridophytic plants having anti-trematodal property is reported first time in this study.

Anthelmintics are drugs that cause adverse effects on the helminths which include the effects on vital activities like feeding, neuromuscular transmission, ion exchange or on the tegument [62]. The normal phytochemicals present in the plant extracts act similar to the mechanisms

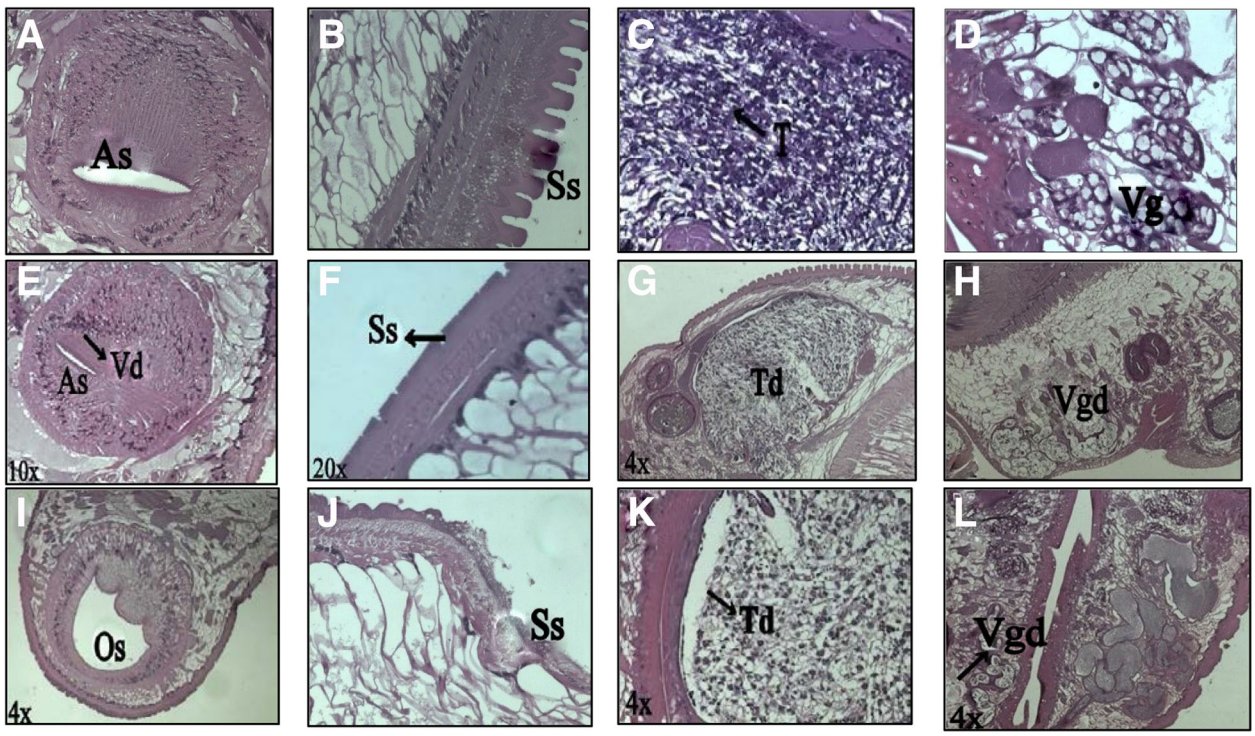

Fig 6. Histopathology study of *Gastrothylax crumenifer*. Histopathology showing (**a**-**d**) Negative control showing normal Anterior Sucker 10x (As) (**a**), Sub Syncytium 10x (Ss) (**b**), Testes 10x (T) (**c**) and Vitelline Gland 10x (Vg) (**d**). **e**-**h** Standard control showing mild vacoular degeneration of Anterior sucker (As) 4x (**e**), Disrupted and detached Sub syncytium (Ss) 10x (**f**), Degeneration of Testes (Td) 10x (**g**), Degeneration of Vitelline gland (Vgd) 4x (**h**). **i**-**l** Test Group, 5 mg/ml conc showing Anterior sucker cell vacuolization (As) 20x (**i**), Detached Surface syncytium (Ss) 10x (**j**), mild Testicular degeneration (Td) 10x (**k**), mild degeneration of Vitelline gland 20x (Vgd) (**l**)

exhibited by conventional anthelmintics. The phytochemical saponins interact with the cell membranes, causing changes within the cell membranes, and subsequent changes in the cell wall [63]. Tannins have the capacity to bind to proteins impair vital process like feeding, reproduction of the parasite and disrupt the integrity of the cuticle [64]. The condensed tannins interact with proline rich proteins on cuticle that will interfere feeding, motility and other key metabolic processes like exsheathment and moulting of the parasites [65].

The effect of *M. fraxinea* Sm. extracts against rumen amphistomes, *G. crumenifer* was tested and it was found that complete paralysis and death of the worm occurred at higher concentration (5 mg/ml) with an incubation time of 60 min, compared to positive control taken 10 min incubation time to kill all worms. Gross microscopical changes under stereo-zoom microscope showed test group of fern worms evinced degenerative changes towards suckers, teguments and testes at higher conc (5 mg/ml). Negative control worms showed smooth spineless tegument followed by surface syncytium, subsyncytial zone and longitudinal and circular muscles, as also observed by [66].

The results of the in vitro incubation study suggests that a dose rate of 1 mg/ml produced negligible changes and 5 mg/ml conc. produced moderate changes, histopathologically in the tegument and affection on muscle integrity when compared to standard drug Oxyclozanide. On microscopic examination, there were effects on the tegument which appeared blebbed, corrugated, bulbous. But the severity of dose rate of 5 mg/ml produced drastic changes in the tegument with blebbing, desquamation, erosion of syncytium and sub-syncytium exposing basal lamina. Histopathological examinations in the present study also suggests affection of the tegument and also the parenchymatous cells, vacoular degenerative changes of Oral sucker which may be depicting an action similar to that of Oxyclozanide where much intense degenerative changes towards testes and ovary noticed. However, the control flukes showed normal microscopic structure of various organs and tegument. The results were in accordance to many other similar works on amphistomes [17, 35, 66].

The major target organ that was highly affected is the tegument and suckers that damages were observed by LM and SEM. The extract effects were less severe than

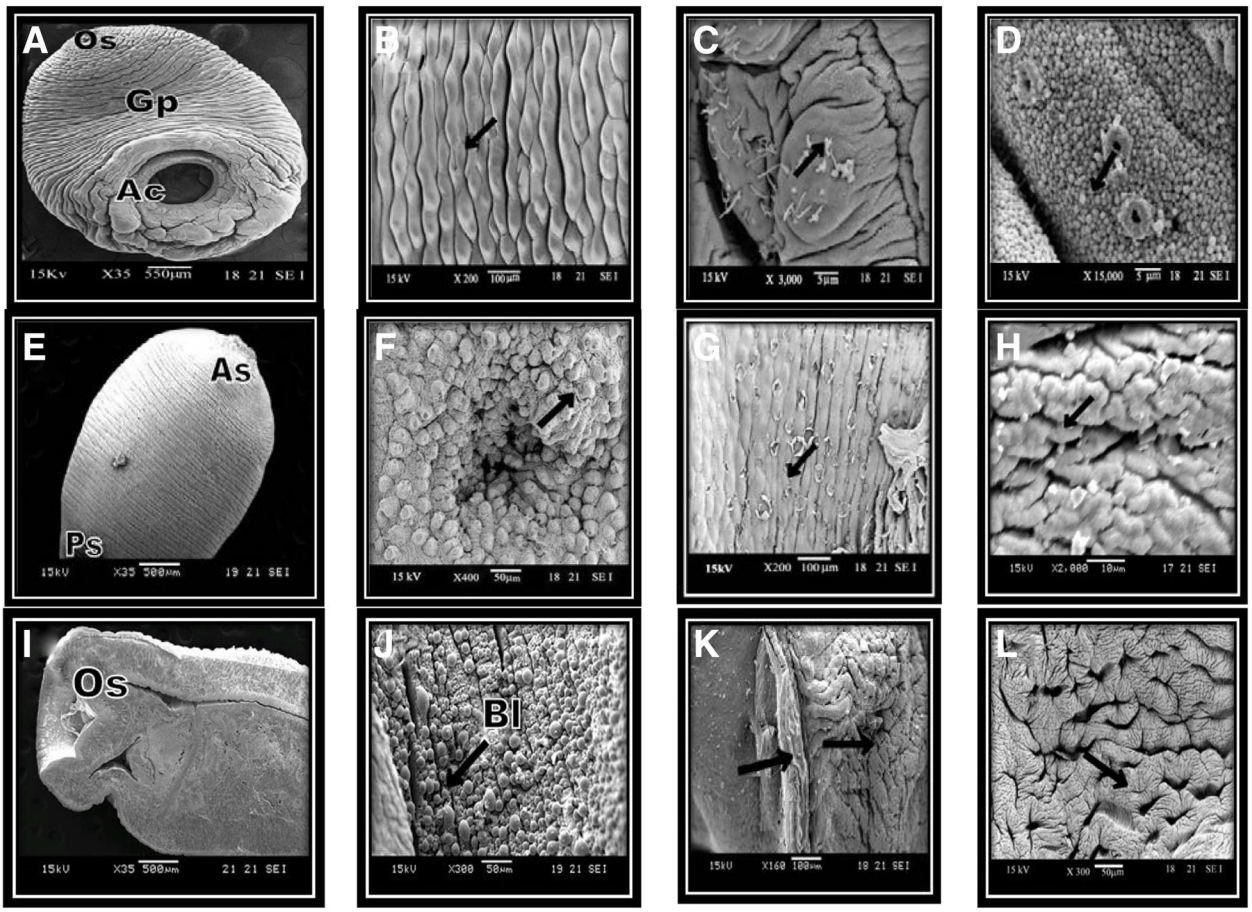

Fig. 7 Scanning Electron Microscopic study of *Gastrothylax crumenifer*. Scanning Electron Microscope (SEMs) of adult *G.crumenifer* incubated in H-F salt solution for 1 h. (**a**-**d**: Negative Control) (**a**) Ventral surface showing Anterior sucker (As), Acetabulum (Ac) and Genital pore (Gp). **b** Ventral surface showing major folds alternate with major grooves. **c** Folds bearing numerous papillae having long cilia. **d** Folds bearing numerous papillae having nipple like tips. (**e**-**h**: Standard Control) (**e**) Dorsal surface, showing Anterior sucker (As) at the anterior tip showing flaccid and retracted changes. **f** Papillae around Anterior sucker (As) showing Blebs. **g** Erosion due to disrupted blebs on tegument surface. **h** Ventral surface showing prominent deep furrows between major folds crossed by minor folds and minor grooves. (**i**-**l**: Test Group) (**i**) Anterior sucker (As) showing degenerative changes at 1 mg/ml conc. (**j**) Severe Blebbing (Bl) on tegumental ventral (5 mg/ml) conc. **k** Deep furrows and major tegumental folds indicating disrupted papillae on ventral surface (5 mg/ml) conc. **l** Middle portion of ventral surface shows prominent Deep furrows (Df) between major folds crisscrossed by minor folds and grooves (5 mg/ml)

Oxyclozanide at high concentration (5 mg/ml) The sequences of tegumental surface changes were similar for all doses of extract compared to OXY, that consisted of swelling, fibrous network formation between major and minor folds, blebbing and subsequent rupture, which lead to erosion and desquamation of the tegument, and finally the exposure and disruption of basal lamina. The tegument is an important structure of parasite because it provides covering and protection of the parasite's body, and supports internal organs. It also controls the secretion, synthesis, perception of sensory stimuli and osmoregulation. It was demonstrated that tegument is a major target of extract, which was probably absorbed by the tegument. The initial tegument swelling was believed to be part of the general response of the fluke to a stress situation, representing an attempt by the fluke to replace damaged surface membrane [67], caused by osmotic imbalance, due to the disruption of ion pumps present on the apical plasma membrane [68]. This was followed by swelling, blebbing, disruption, erosion and lesion. Once the surface layer is totally destroyed, the drug could penetrate deeper into the muscular layer and caused motility reduction and cessation that lead to death. Regional difference of responses to the *M. fraxinea* Sm. extract were also observed, with the ventral being more severely affected than the dorsal surface, and the anterior and middle third regions as well as the lateral margins of the flukes were generally more suffered than the posterior region. The early changes were found at the oral sucker and the genital pore, which exhibited the swollen appearance and scattered blebs along their rims. The acetabulum also was

distorted. Surface changes observed in the present study resembles that demonstrated on *F. gigantica* treated with aqueous extract of *Artocarpus lakoocha* [11], and on *P. Microbothrium* treated with artemether [69].

The tegument of *P. cervi* comprises an outer surface syncytium underlined by a thick subsyncytial zone and musculature [70]. *M. fraxinea* Sm. might be exerted its effect on the tegument first then permeated through the underlying muscle, which exhibited drastically decreased motility. Gross disruption of tegument clearly visible to the naked eye, were observed in all specimens at the higher doses. The fluke's surface appeared dark and followed by tegumental desquamation. On the other hand, the flukes might also ingest MF through the oral sucker because numerous blebs were frequently found at the anterior part of worm, especially around the oral sucker. Dome-shaped papillae are commonly present on trematodes tegument surfaces, and they are believed to have a sensory function [71] related to feeding at the oral aperture, pressure detection on the general body surface, and sexual reception around the genital pore [72]. The papillae were also damaged by *M.fraxinea* Sm. which could cause the loss of sensory functions. Besides, the damage of the acetabulum might affect the holding onto the host tissues [69].

Conclusions

The anthelmintic activity of *M. fraxinea* Sm. extracts against *G. crumenifer*, trematode is perhaps the first report of anthelmintic value of Pteridophytes against trematode infection. The secondary metabolites reported under GC-MS analysis and phytochemical constituents evinced considerable source of terpenoids and polyphenolic derivatives. Terpenoids contribute the highest fraction on quantitative study and acetone extract performed well for qualitative analysis. The in vitro incubation study and Relative Motility (RM) Value suggest that this tree fern extract had reliable source of anti-trematodal property but lesser potent than standard drug oxyclozanide. The anti-trematodal activity of *M. fraxinea* Sm. concluded further with morphology, ultrastructual and histopathological studies, evinced significant changes in teguments and suckers. Hence it is concluded that *M. fraxinea* Sm. can be used as a potent anti-trematodal drug with least side effect, cost effective and to overcome anthelmintic resistance against oxyclozanide and other trematodicidal drugs. However the tegumental enzymes changes could not be analysed in this study, which could be added value to the histochemical property of tremtode-drug action, which could be warranted. Thus knowledge and understanding gained from basic pharmacological research in in vitro and in vivo controlled studies, an array of bioactive molecules could be discovered for further clinical applications in human and veterinary parasitology.

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Authors' contributions
All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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